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Characterization of Mengo Virus Neutralization Epitopes

II. Infection of Mice with an Attenuated Virus

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A panel of five neutralizing monoclonal antibodies was generated from mice immunized with an attenuated strain of Mengo virus. Four of the antibodies were used to select mutants of Mengo virus which were able to escape neutralization by the selecting antibody, but it was not possible to select mutants which could escape neutralization by the fifth antibody. The capsid coding region of the RNA genome of each mutant was directly sequenced to identify the mutation(s) responsible for the neutralization escape phenotype. These results are compared to those of a previous study in which immunogenic determinants recognized by neutralizing antibodies generated against pentameric capsid subunits were located on the external surface of the Mengo virion. We have confirmed the existence of the previously identified immunogenic determinant in VP3 (site 2) as well as an immunodominant determinant in VP2 (site 1). Two previously uncharacterized determinants, located in surface loops of VP1 (sites 3 and 4), were also identified. None of the mutations conferring the neutralization escape phenotype was found near the surface depressions on the virion which are believed to be the receptor binding sites.

INTRODUCTION

Mengo virus is a member of the *Cardiovirus* genus of the Picornaviridae and a natural pathogen of mice. The virion consists of 60 copies of each of four structural proteins surrounding a single-stranded RNA genome of the positive sense. Residues from three of the capsid proteins, VP1, VP2, and VP3, are exposed on the external surface of the virion, while the fourth (VP4) is located on the inner surface of the capsid shell. The structure of the Mengo capsid has been determined to 3-Å resolution (Luo *et al.,* 1987; Krishnaswamy and Rossmann, 1990).

Intraperitoneal injection of Mengo virus in mice results in severe damage to many different cells and tissues, including neurons of the central nervous system and cardiac muscle (Stringfellow *et al.,* 1974). Hindlimb paralysis is the first observable symptom, and the infected mice usually die from severe meningoencephalitis (Veckenstedt, 1974; Guthke *et al.,* 1987). Although rodents are believed to be the natural reservoir of Mengo virus, it has been associated with serious infections of a variety of animals, including pigs (Dea *et al.,* 1991) and primates (Hubbard *et al.,* 1992).

Monoclonal antibodies generated in mice against the 13.4S pentameric subunits derived from dissociated Mengo virions (Mak *et al.,* 1974) have been used to identify some of the capsid surface elements which constitute neutralization epitopes (Boege *et al.,* 1991). Because of the extreme pathogenicity of wild-type Mengo virus, immunization of mice using intact infectious virions for the generation of monoclonal antibodies is not possible. However, Duke and Palmenberg (1989) generated a cDNA clone of Mengo virus that contained a truncated poly(C) tract in the 5′ noncoding region of the RNA genome, and Duke *et al.* (1990) showed that this clone produced viruses which were attenuated for mice. This Mengo strain has recently been used as a vector for live recombinant vaccines (Altmeyer *et al.,* 1994, 1995), and we have used it as an immunogen to further the characterization of Mengo virus B-cell epitopes recognized by the mouse.

B-cell epitopes of a large number of picornaviruses have been mapped using monoclonal antibodies generated from immunized mice. These include poliovirus (genus *Enterovirus:* relevant recent publications are Hogle and Filman, 1989; Weigers *et al.,* 1990; Roivainen *et al.,* 1991; Reynolds *et al.,* 1991; Patel *et al.,* 1993), Coxsackie B virus (genus *Enterovirus:* Haarmann *et al.,* 1994), bovine enterovirus (genus *Enterovirus:* Smyth *et al.,* 1990), human rhinoviruses 2 and 14 (genus *Rhinovirus:* Rossmann *et al.,* 1985; Sherry *et al.,* 1986; Speller *et al.,* 1993), foot and mouth disease virus (genus *Aphthovirus:* Saiz *et al.,* 1991; Crowther *et al.,* 1993), human hepatitis A virus (genus *Hepatovirus:* Ping and Lemon, 1992), and Theiler's murine encephalitis virus (genus *Cardiovirus:* Kim *et al.,* 1992; Roos and Casteel, 1992). However, with the excep-

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tion of the Theiler's virus, these viruses do not productively infect mice and the murine B-cell response to these viruses may not be entirely representative of the protective response which occurs in their natural hosts.

We have examined the B-cell response generated in mice to infectious, attenuated Mengo virus and identified a number of monoclonal antibodies which specifically recognize and neutralize the virus. The epitopes recognized by most of these monoclonal antibodies were identified by sequencing of naturally occurring neutralization escape mutants and compared with those identified in our earlier study (Boege *et al.,* 1991), which used noninfectious subviral pentamers as immunogen.

METHODS

Production and purification of Mengo virus

Confluent mouse L-929 cell monolayers in roller bottle cultures were infected with Mengo virus (M plaque variant; Ellem and Colter, 1961) at low multiplicity of infection (0.1 plaque-forming unit, PFU, per cell). Purified Mengo virus was prepared from tissue culture supernatants as described previously (Ziola and Scraba, 1974; Boege *et al.,* 1986).

Hybridoma production for anti-Mengo virus monoclonal antibodies

BALB/c mice were initially injected, by the intraperitoneal (ip) route, with 5×10^6 PFU of purified attenuated Mengo virus (Duke *et al.,* 1990; generously provided by Dr. A. C. Palmenberg). Mice were given three ip booster injections of 7.5 μg of purified wild-type Mengo virus at 30-day intervals, and those with a serum ELISA titer against Mengo virus of greater than 1:1000 were sacrificed 3 days after the final injection. Hybridomas were formed by PEG (PEG 3350, Sigma P-2906)-mediated fusion of splenocytes with SP2/0 cells combined at a ratio of 8:1, respectively, and distributed into 96-well tissue culture plates. Hybridomas positive for the production of anti-Mengo virus-specific antibodies were identified by ELISA and cloned by limiting dilution. All monoclonal cell lines selected after one cycle of cloning were recloned to ensure that they were monoclonal.

Enzyme-linked immunoadsorbant assays (ELISA)

High protein affinity polystyrene ELISA plate (Immulon 2, "U" plates, Dynatech Laboratories Inc.; supplied by Fisher) wells were coated with purified Mengo virus (3 μg/ml, 100 μl/well) overnight at 4° in ELISA-coating buffer (13.5 m*M* Na_2CO_3, 34.9 m*M* $NaHCO_3$, pH 9.6). Each well was treated with 200 μl of 1% BSA (Sigma, fraction V, A-4503) in PBS for 1 hour at 20°. One hundred microliters of either undiluted hybridoma supernatant or ascites fluid diluted in PBS–Tween was added to each well and the plates were incubated for 2 hr at 20°. To detect antibody bound to the virion antigen, plates were incubated for 1.5 hr with a 1:1500 dilution (100 μl/well) of alkaline phosphatase-conjugated goat anti-mouse IgA-, IgG-, and IgM-specific antibody mixture (Cedarlane Laboratories, Hornby, Ontario). Bound alkaline phosphatase-conjugated antibody was detected by the addition of 100 μl/well of substrate [5 mg *p*-nitrophenyl phosphate (Sigma 104-105) in 5 ml 10% diethanolamine, pH 9.8]. Positive results were quantitated at 410 nm with an ELISA plate reader (MR600 Micro Plate Reader, Dynatech Laboratories Inc.).

Determination of monoclonal antibody isotype

ELISA plates were coated with Mengo virus as in the ELISA procedure. The antibody type in undiluted hybridoma tissue culture supernatant or ascites fluid diluted in PBS was determined with the Bio-Rad Mouse Typer Isotyping kit (172-2051) according to the instructions provided.

Microneutralization and cross-neutralization assays

The resistance of each mutant virus isolate to each of the neutralizing monoclonal antibodies was tested in a microtiter neutralization test. In each well, 50 μl of undiluted hybridoma supernatant was incubated with 50 μl of mutant isolate at 100-fold 50% tissue culture infective dose for 1 hr at 37°, and then 5×10^4 L-929 cells, in 100 μl basal medium Eagle supplemented with 10% FCS, was added. After 48 hr at 37°, the plates were stained with 0.1% crystal violet in 20% ethanol or examined by light microscopy to detect cell lysis.

Western blots

Mengo virus capsid proteins (50 μg/lane) were separated by electrophoresis in a 1-mm, 7.5% SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose (Bio-Rad 162-0115) by wet electrophoretic transfer [10 m*M* CAPS (3-(cyclohexylamino)-1-propanesulfonic acid), 10% methanol, pH 11.0] for 15–18 hr at 60 V and 4°. Transfer was checked by staining the blot with 0.1% Coomassie brilliant blue in 50% methanol and destaining with 50% methanol and 10% acetic acid. The blot was blocked with 3% BSA in PBS for 2 hr at 20° and then washed with four changes of 150 m*M* NaCl, 50 m*M* Tris (pH 7.5). The bound alkaline phosphatase-conjugated antibodies were detected using bromochloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT). The substrate solution used was 66 μl NBT stock (5% NBT in 70% dimethylformamide) plus 33 μl BCIP stock (5% BCIP in 100% dimethylformamide) in 10 ml alkaline phosphatase buffer (100 m*M* NaCl, 5 m*M* $MgCl_2$, 100 m*M* Tris, pH 9.5). The blot was developed until the appearance of bands and the reaction was stopped by replacing the substrate with PBS containing 20 m*M* EDTA.

Selection of mutant Mengo virions which escape neutralization

The procedure used for the selection of mutants with antibodies 370, 9B, and 352 has been described (Boege

et al., 1991). Mutants which escape neutralization by antibody 3A could not be selected from the wild-type stock of Mengo virus, but could be selected by the same procedure using a stock of mutant virus 4/12 (selected for its ability to escape neutralization by monoclonal antibody MCP4; Boege *et al.,* 1991), which has a lysine to threonine mutation at position 2148 in VP2. It was not possible to select mutants which escape neutralization by antibody 6A from either wild-type Mengo virus or stocks of any of the mutants of Mengo virus selected to escape neutralization by other monoclonal antibodies (MAbs). The actual frequencies of mutation were not determined for each mutant, but all were in the range (10^{-3} to 10^{-5}) which had been calculated for mutations escaping neutralization by anti-pentamer MAbs (Boege *et al.,* 1991; Table 1).

RNA extraction from mutant viruses

Pellets of mutant virus obtained from 6×10^8 infected cells were resuspended in 2.25 ml RNA buffer (100 m*M* KCl, 10 m*M* Tris, 1 m*M* EDTA, pH 7.2, containing 1% SDS) and extracted three times with equal volumes buffer-saturated phenol:chloroform (4:1). After three washes of the aqueous phase with buffer-saturated ether and removal of residual ether by N_2, 200 μl of 2 *M* sodium acetate (pH 5.0) was added and the RNA precipitated with three volumes of cold (−25°) absolute ethanol. The pellet was recovered by centrifugation, dried under vacuum, resuspended in 50 μl water, and stored at −25°.

RNA sequence determination

This procedure has been described previously (Boege *et al.,* 1991).

cDNA synthesis and cloning

Neutralization escape mutants selected with antibody 3A do not replicate efficiently in culture so it was not possible to obtain sufficient mutant virus to isolate RNA. Sequences of the mutants were determined from PCR-amplified cDNA produced from cytoplasmic RNA in infected cells. Cytoplasmic RNA was purified from Mengo virus-infected mouse L-cells by the method of Sambrook *et al.* (1989). cDNA from the capsid coding region was synthesized using 1 μg of RNA with primer P27 (5′-TAGCAGGATGTGCAGAGT-3′) and 200 units of Superscript reverse transcriptase (GIBCO BRL). The cDNA was amplified by PCR with addition of primer P7 (5′-CAGTCAACGACTCCTCAGGGT-3′) which binds outside the capsid coding region of the genome. The 2640-bp fragment was excised from an agarose gel, purified with GeneClean (Bio101), and ligated into the *Eco*RV-digested pBluescriptII (SK^+) (Stratagene) using standard techniques (Sambrook *et al.,* 1989).

TABLE 1

Antibody Typing of Various Monoclonal Antibody-Producing Cell Lines

Monoclonal antibody	Immunoglobulin class
370	IgM
3A	IgA
6A	IgG_{2A}
9B	IgM
352	IgM

DNA sequencing

Plasmids containing inserts were purified using Qiagen columns (Qiagen Inc.) and then sequenced using a PRISM Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems) with primers covering the capsid region. Reactions were processed with an Applied Biosystems 373A automated DNA sequencer and analyzed using the Applied Biosystems Seqed program.

Ribbon diagrams

The ribbon diagrams of Mengo capsid proteins VP1, VP2, and VP3 were generated from the crystallographic coordinates for the virus (Brookhaven Protein Data Bank) using the graphics program MOLSCRIPT (Kraulis, 1991).

Nomenclature

Amino acid residues are identified by their position in the polypeptide sequence preceded by 1, 2, 3, or 4 depending on respective capsid protein VP1 through VP4.

RESULTS

Five monoclonal cell lines which produced neutralizing antibody against Mengo virus were generated by fusions of splenocytes from mice immunized with the attenuated Mengo virus with myeloma cells. The cell lines were initially identified using ELISA to screen for the presence of virus-specific secreted antibody and then screened in microneutralization assays to identify which were capable of neutralizing Mengo virus infectivity *in vitro.* The names and immunoglobulin class of the MAbs secreted by these hybridoma cell lines are listed in Table 1.

Neutralization escape mutants were selected from wild-type Mengo virus stock using MAbs 370 (mutants 370/1-L, 2-L, 5, and 8-L), 9B (mutants 9B/2, 3, 4, and 9), and 352 (mutants 352/1, 5, 6, and 7). It was not possible to select mutants from wild-type Mengo virus which were capable of escaping neutralization by either MAb 6A or 3A. However, it was observed during characterization of these two antibodies that mutants which did escape neutralization by 3A could be selected from pools of mutants which had previously been selected for their ability to escape certain anti-Mengo virus MAbs which had been generated from mice immunized with pentameric subunits (see below). Neutralization escape mutants

could be selected from pools of all mutants selected with MCP4 or from mutant 6/16. Four mutants (3A/8, 9, 10, and 11) were selected from mutant virus 4/12 stock with antibody 3A for further characterization. It was not possible to select neutralization escape mutants with MAb 6A from either wild-type Mengo virus stock or stocks of any of the other available mutants of Mengo selected with other anti-Mengo antibodies.

Initial characterization of the antibodies and mutants was done with a series of cross-neutralization assays in which the ability of each antibody to neutralize the mutants selected with all of the other antibodies was tested (Fig. 1). These assays included the mutants and antibodies described in Boege *et al.* (1991). MAb 6A, for which no neutralization mutants could be selected, completely neutralized the infectivity of wild-type virus and all the mutants.

MCP4 neutralized all mutants viruses except 5/01-S, 5/01-M, 5/01-L, 6/16, and the mutants selected with antibody 3A. MCP5 neutralized all mutants except those selected with antibody 3A. MCP6 did not neutralize mutants 5/01-S, 5/01-M, and 5/01-L or the mutants selected with antibodies MCP4 and 3A. Although MAb 3A neutralized all the mutants selected with other antibodies, it was possible to select neutralization escape mutants to MAb

CROSS-NEUTRALIZATION DATA

	Monoclonal Antibody								
Mutant Virus	MCP1	MCP4	MCP5	MCP6	370	3A	6A	9B	352
1/01	□	■	■	■	■	■	■	□	■
1/02	□	■	■	■	■	■	■	■	■
1/03	□	■	■	■	■	■	■	■	■
1/04	□	■	■	■	■	■	■	■	■
1/06	□	■	■	■	■	■	■	■	■
1/11	□	■	■	■	■	■	■	■	■
4/01	■	□	■	□	■	■	■	■	■
4/02	■	□	■	□	■	■	■	■	■
4/04	■	□	■	□	■	■	■	■	■
4/08	■	□	■	□	■	■	■	■	■
4/09	■	□	■	□	■	■	■	■	■
4/12	■	□	■	□	■	■	■	■	■
5/01-S	■	□	□	□	■	■	■	■	■
5/01-M	■	□	□	□	■	■	■	■	■
5/01-L	■	□	□	□	■	■	■	□	■
5/04	■	■	□	■	■	■	■	■	■
5/11-S	■	■	□	■	■	■	■	■	■
5/11-L	■	■	□	■	■	■	■	□	■
6/16	■	□	■	□	■	■	■	■	■
6/18	■	■	■	□	■	■	■	■	■
370/1-L	■	■	■	■	□	■	■	■	■
370/2	■	■	■	■	□	■	■	■	■
370/5-L	■	■	■	■	□	■	■	■	■
370/8-L	■	■	■	■	□	■	■	■	■
3A/8	■	□	□	□	■	□	■	■	■
3A/9	■	□	□	□	■	□	■	■	■
3A/10	■	□	□	□	■	□	■	■	■
3A/11	■	□	□	□	■	□	■	■	■
9B/2	■	■	■	■	■	■	■	□	■
9B/3	■	■	■	■	■	■	■	□	■
9B/4	■	■	■	■	■	■	■	□	■
9B/9	■	■	■	■	■	■	■	□	■
352/1	■	■	■	■	■	■	■	□	□
352/5	■	■	■	■	■	■	■	■	□
352/6	■	■	■	■	■	■	■	■	□
352/7	■	■	■	■	■	■	■	■	□

FIG. 1. Cross-neutralization pattern showing the anti-Mengo virus monoclonal antibodies and the escape mutants. Mutants selected with one monoclonal antibody were tested for the ability to escape the other monoclonal antibodies; □ represents mutants which escaped neutralization; ■ represents neutralization of the mutant.

3A starting with mutant 6/16 or the mutants selected with antibody MCP4 but not from wild type or any other mutant virus stock (Boege *et al.,* 1991; see Fig. 1). This suggested that MAb 3A binds to an epitope which includes the site of the mutations in these escape mutants. Together these results indicate that the epitopes recognized by MAbs MCP4, 5, 6, and 3A overlap extensively, are distinct from the epitopes recognized by the other MAbs, and together define one antigenic determinant, termed site 1, on the Mengo virion (Table 3).

MCP1 was found to be capable of neutralizing mutants selected with all of the other antibodies. This is in contrast to the results we originally reported, which indicated that MCP1 would not neutralize the mutants selected with the other anti-pentamer antibodies MCP4, 5, and 6 (Boege *et al.,* 1991). This error likely resulted from use of insufficient antibody in the cross-neutralization tests. MAbs 352 and 370 neutralized all the mutants selected with the other antibodies, and none of the mutants selected with either MAb were neutralized by any other antibody. None of the mutants selected with MAb 9B escape neutralization by the other MAbs although 9B could not neutralize mutants 1/01, 5/01-L, 5/11-L, and 352/1.

The precise locations of the epitopes recognized by the antibodies in the virus capsid were identified by complete sequencing of the region of each mutant genome coding for capsid proteins VP1, VP2, and VP3. For mutants selected with antibodies 370, 9B, and 352, the RNA genomes were sequenced directly using 12 different synthetic oligonucleotide primers designed to cover the region of interest. It was necessary to use an alternative approach to map the mutations in mutants selected with antibody 3A because these mutants did not produce significant amounts of virus when grown in culture, even though they were able to infect cells. To determine the position of neutralization escape mutations in the capsid of these viruses, cDNA was produced from Mengo virus RNA isolated from infected cells, amplified by the polymerase chain reaction, and sequenced in the capsid coding region with the primers used for RNA sequencing. Table 2 indicates the location of the nucleotide mutation(s) found for each mutant and the resulting amino acid changes in the capsid proteins. The mutants selected with MAb 370, designated 370/1-L, 2-L, 5, and 8-L, all have a mutation at residue 1100 in VP1. The MAb 9B-selected mutants (9B/2, 3, 4, 9) all have a mutation resulting in a change in either residue 1049 or 1062 in VP1, and mutant 9B/4 has an additional mutation at residue 2016 in VP2. The latter is a secondary mutation and is not likely to be involved in the neutralization escape phenotype of the mutant: residue 2016 is buried in the interior portion of the capsid protein and mutants 9B/2 and 9B/9 also have mutations at residue 1062 which alone are sufficient to confer neutralization resistance to MAb 9B. The mutants selected with MAb 352 (352/1, 5, 6, 7) have mutations at residues 3073, 3074, and 3075 in

TABLE 2

Changes in Nucleotide Sequence and Amino Acid Sequence of Mengo Virus Escape Mutants

Mutant virus	Nucleotide change[a]	Amino acid change[b]		
		VP1	VP2	VP3
370/1-L	2928 A → G	1100 Thr → Ala		
370/2-L	2929 C → U	1100 Thr → Ile		
370/5	2929 C → U	1100 Thr → Ile		
370/8-L	2929 C → U	1100 Thr → Ile		
9B/2	2814 A → G	1062 Asn → Asp		
9B/3	2775 A → C	1049 Lys → Gln		
9B/4	1217 A → C		2016 Gln → His	
	2814 A → C	1062 Asn → His		
9B/9	2814 A → G	1062 Asn → Asp		
352/1	2775 A → C	1049 Lys → Gln[c]		
	2154 A → C			3073 Lys → Gln
352/5	2160 C → G			3075 Gln → Glu
352/6	2158 C → U			3074 Thr → Ile
352/7	1600 A → G		2144 Asn → Ser[c]	
3A/8[d]	1599 A → G		2144 Asn → Asp	
3A/9	1599 A → G		2144 Asn → Asp	
	2347 C → U			3137 Thr → Ile
3A/10	1599 A → G		2144 Asn → Asp	
3A/11	1599 A → G		2144 Asn → Asp	

[a] The nucleotide numbers refer to the position in the wild-type Mengo virus genome, beginning with nucleotide 1 at the 5′-end [the complete poly(C) tract is included].

[b] The amino acid residues are identified according to the "Rossmann system," with the first digit representing viral capsid protein 1, 2, or 3, and the next three digits indicating position from the N-terminal.

[c] The mutation at residue 1049 in escape mutant 352/1 is probably a second site mutation. The mutation at 2144 indicates that antibody 352 may span sites 1 and 2 (Table 3; see text).

[d] Escape mutants for antibody 3A were selected from a stock of virions which already contained a Lys → Thr mutation at residue 2148.

VP3 or at residue 2144 in VP2. Mutant 352/1 has an additional mutation at residue 1049 in VP1, which is probably a secondary mutation. However, this mutation, as well as secondary mutations in mutants 1/01, 5/01-L, and 5/11-L at residues 1049 or 1062, confers neutralization resistance to MAb 9B (Fig. 1).

All of the mutants selected with MAb 3A have a lysine to threonine mutation at residue 2148 of VP2 of the starting mutant 4/12 virus stock. Mutant 3A/9 was completely sequenced and two additional mutations were identified in the capsid proteins at residue 3137 of VP3 and at 2144 of VP2. Sequencing in these regions of mutants 3A/8, 10, and 11 showed that all had the same mutation at residue 2144 but none of the other mutants had a change at residue 3137.

The epitope recognized by MAb 6A could not be identified by the selection and sequencing of neutralization escape mutants. Alternative methods were attempted to characterize the binding site of MAb 6A and the Mengo virion. Western blot analyses of this antibody against the individual capsid proteins of the virus indicate that it specifically recognized VP2 (Fig. 2). Additional attempts to characterize its binding site by direct binding of antibody to overlapping peptides (Bolwell *et al.,* 1989) representing all of capsid protein VP2 and competition binding assays (Parry *et al.,* 1989) using the same peptide panel to compete for binding to virions were not successful.

Based on the patterns of cross-neutralization and the sequencing data, four B-cell determinants on the Mengo virion have been identified. The results are summarized in Table 3. Our previous study (Boege *et al.,* 1991) which mapped the B-cell determinants of the Mengo virion using monoclonal antibodies against pentameric subunits of the virion identified two separate determinants, site 1 and site 2. Site 1, recognized by MAbs MCP4, 5, and 6, includes residue 2075 in the βB–βC loop of VP2 (Luo *et al.,* 1987) and residues 2144, 2145, 2147, and 2148 in the

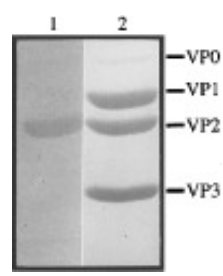

FIG. 2. Western blot analysis of Mengo virus capsid proteins using monoclonal antibody 6A (lane 1). Lane 2 contains the major Mengo virus capsid proteins stained with Coomassie brilliant blue.

TABLE 3

B-Cell Determinants on the Mengo Virion

Antigenic (neutralization) site[a]	Recognized by monoclonal antibody[b]	Escape mutants	Location in the virus capsid[c]
1	MCP4, MCP6	K2075	VP2: loop between βB and βC
	MCP4, MCP5, MCP6, 3A	N2144 R2145	VP2: surface "puff" between βE and αB
	MCP4, MCP6	S2147 K2148	
2	MCP1	K3057	VP3: surface "knob" inserted in βB
	MCP1	A3061	
	MCP1	S3068	
	352[d]	K3073	VP3: loop between βB and βC
		T3074	
		Q3075	
3	370	T1100	VP1: loop II between βC and αA
4	9B	K1049	VP1: carboxyl-end of βB
	9B	N1062	VP1: loop between βB and βC

[a] The anti-pentamer MAbs MCP4, MCP5, and MCP6 have been assigned to site 1 and MCP1 assigned to site 2 based on corrected data from Boege *et al.* (1991).

[b] Antibodies MCP4, MCP5, and MCP6 eere raised using pentamers; 352, 9B, 3A, and 370 were raised using attenuated Mengo virus.

[c] See Luo *et al.* (1987) and Fig. 3.

[d] Cross-neutralization data (Fig. 1) do not indicate that the epitopes recognized by antibody 352 and MCP1 are in the same site; however, the escape mutations from both are in close proximity on the surface of the virion (Fig. 4).

βE–αB loop of VP2. Site 2, recognized by MCP1, includes residues 3057 and 3061 in the surface "knob" inserted in βB of VP3 as well as residue 3068 in the βB–βC loop of VP3. In this study antibodies which bind to sites 1 and 2 as well as to two previously unidentified epitopes on the virion have been characterized. MAb 3A recognizes site 1, while MAb 352 may overlap sites 1 and 2 and probably expands the residues recognized as part of site 2 to include 3073–3075 in the βB–βC loop of VP3. Site 3, recognized by MAb 370, includes residue 1100 in loop II between the βC strand and αA helix of VP1. Site 4 contains residues 1049 and 1062 located in the βB–βC loop of VP1 and is recognized by MAb 9B (Fig. 3).

The relative positions of the four immunogenic determinants of the Mengo virion and the "pit" are shown on the schematic diagram in Fig. 4. The pit is believed to be the site of interaction of the virion with its cellular receptor (Luo *et al.,* 1987; Rossmann and Palmenberg, 1988) and, as expected, none of the antigenic determinants involve residues located in or near the pit. Such mutations would not be viable since they would destroy the receptor binding ability of the virus.

DISCUSSION

In a previous study two antigenic determinants of Mengo virus (sites 1 and 2; Table 3) were identified using MAbs generated against pentameric subunits (Boege *et al.,* 1991). The analysis of antigenic determinants has been continued using five hybridoma cell lines, which secrete neutralizing MAbs, that were generated from mice immunized with infectious attenuated Mengo virus. Four of the antibodies were used to select naturally occurring neutralization-resistant mutants but mutants could not be selected which could escape the fifth antibody (MAb 6A). The capsid protein coding region of the genome of each mutant was sequenced to identify the mutation(s) responsible for the ability of the mutant to escape its selecting antibody. The positions of these mutations were then mapped on the three-dimensional structure of the virion (Luo *et al.,* 1987; Krishnaswamy and Rossmann, 1990). The results are summarized in Table 2 and Fig. 3.

Antivirion MAb 3A was shown to recognize an epitope containing residues within site 1. MAb 3A is of interest because a single mutation within its epitope did not confer neutralization resistance. Neutralization escape mutants could be selected from virions with a preexisting amino acid mutation (lys → thr at residue 2148) within site 1. All mutants so selected were found to contain an identical additional mutation at residue 2144, suggesting that MAb 3A has a sufficiently high affinity for its epitope and that at least two mutations within its virion binding site are required to abrogate recognition. The mutations must also occur at specific residues because not all mutants which had a single mutation within site 1 could be used as a starting stock from which to select neutralization escape mutants. To our knowledge, this is the first example of a picornavirus neutralizing MAb that requires more than one mutation in its viral epitope for escape from neutralization.

MAb 6A is another example of an antibody which is able to neutralize Mengo virus very efficiently, since no escape mutants could be isolated for this antibody, even when other neutralization escape mutants were used as starting stocks. It was possible to show an interaction of MAb 6A with VP2 polypeptides by Western blotting. MAb 6A may also require multiple mutations within its binding site for virions to escape neutralization. Functional studies have demonstrated that epitopes may contain far fewer amino acid residues which are necessary for specific recognition than the total number of residues which are in the contact region of the antibody (Geysen *et al.,* 1984; Getzoff *et al.,* 1987; Jin *et al.,* 1992). This would suggest why most neutralization escape mutants selected with a given MAb gener-

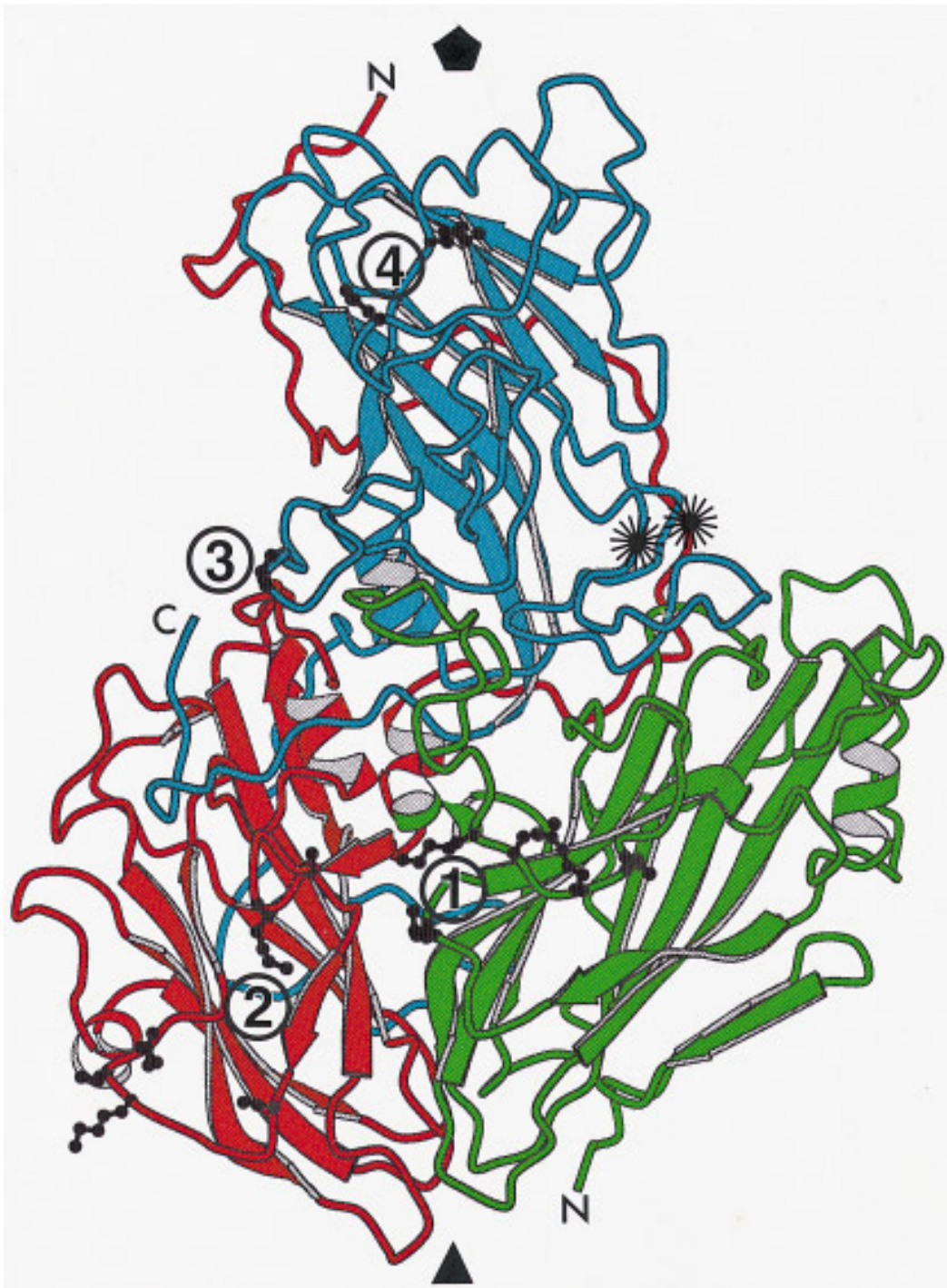

FIG. 3. Ribbon diagram of the biological protomer of Mengo virus showing the location of the four antigenic sites defined by "escape" mutants. VP1 is blue, VP2 is green, and VP3 is red. The letter C designates the last residue visible in the original electron density map for VP1 (P1259); amino-termini of VP2 and VP3 are indicated with the letter N. Ball-and-stick representations of the amino acid residues defining sites 1 (K2075, N2144, R2145, S2147, and K2148), 2 (K3057, A3061, S3068, K3073, T3074, and Q3075), 3 (T1100), and 4 (K1049 and N1062) are shown in black. The location of the "pit," the putative receptor-binding site (Luo *et al.,* 1987), is indicated by the sunburst symbols. The five- and threefold axes of the virion are marked by the black symbols to show the orientation of the biological protomers in the capsid structure.

ally require only a single mutation, often occurring at the same critical residue in all mutants, to escape recognition. Both MAb 3A and (perhaps) MAb 6A are unusual in this regard because a single mutation cannot confer neutralization resistance. Such antibodies would be advantageous for the infected host because it is highly unlikely that multiple mutations will occur simultaneously in a given virion within the binding site of a single monoclonal antibody.

The epitope recognized by MAb 352 is enigmatic. Escape mutants 352/1, 352/5, and 352/6 have single amino

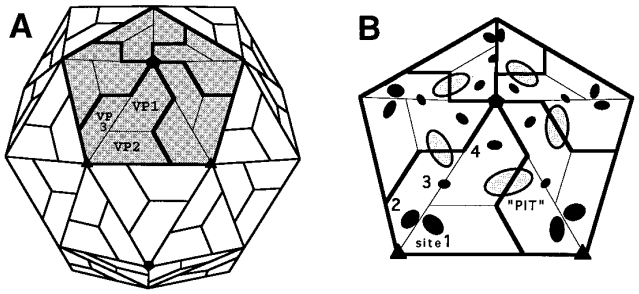

FIG. 4. (A) Diagrammatic representation of the Mengo virus capsid. The capsid is assembled from five pentamers, one located at each of the 12 vertices of a regular icosahedron. The biological protomers (VP1, VP2, VP3) which comprise one pentamer (gray) are outlined with thick black lines. (B) A single pentamer showing the locations of antigenic sites 1–4. None of these are located in close proximity to the surface depression ("pit") which is thought to be the binding site for a cellular receptor.

acid changes in the βB–βC loop of VP3 (the K1049Q mutation in 352/1 is likely not involved in neutralization by the antibody). We have tentatively assigned these mutations to site 2 because of their proximity to those residues in the epitope recognized by MCP1 (Fig. 4), but this assignment was not confirmed by the cross-neutralization experiments (Fig. 1). The N2144S mutation was the only one found in mutant 352/7. It is conceivable that this could be a second site mutation, with the real escape mutation remaining undetected. Alternatively, the epitope recognized by MAb 352 may in fact span sites 1 and 2. The closest approach of these residues would be between the VP3 residues of one pentamer and the VP2 residues from an adjacent protomer across the icosahedral twofold axis; this suggests that this epitope may comprise surface residues from two adjacent pentamers. The distance between these residues on the surface of a single protomer would approach the 30-Å limit of the diameter of an antibody footprint (Davies *et al.,* 1990).

In addition to confirming the existence of sites 1 and 2, originally identified with the anti-pentamer MAbs, two additional neutralization determinants have been identified with the anti-virion MAbs. Site 3 is defined by the epitope recognized by MAb 370 and site 4 is defined by the epitope recognized by MAb 9B (Table 3). As in sites 1 and 2, the mutations in neutralization-resistant mutants selected with these MAbs are located on the surface of the capsid proteins, again suggesting that mutant Mengo viruses avoid the neutralizing activity of the antibody by directly altering the binding site of the antibody. Some mutations in foot-and-mouth disease virus and poliovirus at sites not directly accessible to antibody binding can confer neutralization resistance by the disruption of hydrogen bonds or salt bridges which cause a local disturbance in the conformation of an antigenic loop (Page *et al.,* 1988; Parry *et al.,* 1990; Krebs *et al.,* 1993). Mutations causing conformational disruption of an epitope at a site distant from the epitope have not been observed in picornaviruses, presumably because such changes would have a deleterious effect on capsid stability and/or function.

Neutralization epitopes of Mengo virus were analyzed by Muir *et al.* (1991) using a peptide-based system. They tested the ability of antisera generated in mice against each of 140 overlapping synthetic peptides, covering all of the sequences of the four capsid proteins, to neutralize Mengo virus. Only one of these peptides, corresponding to residues 1259–1277 of the external and flexible carboxyl-end of VP1, induced significant levels of polyclonal neutralizing antibodies. This epitope was not identified in our experiments using either subviral pentamers or live attenuated virus as immunogen. Muir *et al.* (1991) also reported that three other peptides in VP1 (residues 1041–1050, 1081–1090, and 1163–1172) induced a very weak neutralizing response against Mengo virus. One of these peptides contains residue 1049 that is part of the epitope recognized by MAb 9B.

The three-dimensional structure of the Mengo capsid (Luo *et al.,* 1987) revealed a number of linear amino acid sequences predicted to be candidates for immunogenic epitopes. These sites include the two loops between βB and αA regions in VP1 (residues 1078 to 1083 and 1093 to 1105) and the prominent βE–αA loop in VP2 (residues 2154 to 2165). Of these sites, only the loop in VP1 contained a neutralizing immunogenic epitope (recognized by MAb 370; Table 3). While it may be possible that we have not examined a sufficient number of antibodies to identify these other sites, it is clear from the current results that neutralizing antibodies recognize regions of the capsid exposed on the surface but not necessarily on prominent protrusions. It is possible that more specific residues within the antibody binding site are required for stable binding than are critical for the initial recognition of the epitope (Jin *et al.,* 1992), and an extended loop may not provide enough surface contacts for stable binding. Second, it is not unreasonable to speculate that host antibodies are produced against all parts of the surface of the virus, including the highly accessible loops, but binding to these loops may not provide the required antibody–antigen interaction for neutralization of virus infectivity. In this context, peptide vaccines designed solely on the surface accessibility of linear determinants may be ineffective; additional factors must be considered in determining whether a particular amino acid sequence can elicit a pathogen-neutralizing antibody response.

In a recent review Lea and Stuart (1995) used computer graphics techniques to analyze the external surfaces of picornavirus (HRV-14, FMDV-O_1, poliovirus-1, and Mengo virus) capsids with respect to exposed amino acid residues. Comparison with a control group of proteins revealed that the surfaces of picornavirus capsids were particularly rich in threonine residues ($\sim$21% of the surface area as compared to $\sim$4% for control proteins). This observation led Lea and Stuart to suggest that threonine residues would be evolutionarily favored for the surfaces of proteins which depend on antigenic polymor-

phism for their survival. The rationale presented for this hypothesis is that a transition mutation in either of the first two positions of the threonine codons (AC-) would yield alanine, isoleucine, or methionine. This change could result in the replacement a polar serine residue which contributed specific recognition to an epitope (by hydrogen bonding with the antibody combining site) to a residue "not provoking strong interactions with immunoglobulins." This they suggested, would "render the virus less visible to the host immune system." Inspection of the Mengo capsid surface (Rossmann and Palmenberg, 1988) shows that ~13% of the exposed residues are threonines (24 out of a total of 183 in each capsid protomer). Of the 14 separate mutations which we have found to result in escape from neutralization by a monoclonal antibody, only 2 involve threonine residues. Both of these fulfill the Lea and Stuart criterion in that the escape mutants all have either isoleucine or alanine as replacements; no third-position mutations were found. However, the majority of the neutralization mutations follow no recognizable pattern with respect to transitions vs transversions, position of the mutation within the codon, or replacement of polar/charged with nonpolar/uncharged residues.

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